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Crispr/Cas9 Endonucleases: A New Era of Genetic Engineering

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Abstract

In modern genetic engineering, there is always development towards better ways for therapies of different diseases in the most efficient way. Genetic engineering approaches use the nucleases to cut the DNA. Meganucleases, ZFN, TALEN and CRISPR i.e. clustered regularly interspaced short palindromic repeats determine PAM sequence. These sequences are present as direct repeats that are separated by specific Spacers and have Cas genes which are present adjacent to the spacer regions. Microbe's immunity is related to the presence of CRISPR sequences, which can cleave and bind the DNA at specific sequences. CRISPR is classified into two classes that are further divided into 5 types. Most commonly used class is type 2 which work along the CRISPR associated protein called Cas 9 obtained from *Streptococcus pyogene*. Cas 9 is a multi-subunit protein with two nuclease domains called HNH and RuvC like domains. The presence of a smaller sequence upstream to the DNA that is to be targeted is important for specific cleavage and is called the Seed Sequence. CRISPR have many applications in genome editing and beyond genome editing.

Keywords: AML; Pathogenesis; Treatment; Relapse

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1. INTRODUCTION

In modern genetic engineering, there is always development towards better ways for therapies of different diseases in the most efficient way. Manipulation of gene carriers i.e. DNA and RNA aim to either add, delete or modify the information carried by the carriers in such a way that either the disease can be cured or prevented by even happening [1].

Editing of the genome has been proven important in many fields and thus is one of the most researched zones to introduce or cut any of the genes of interest to work out the result of interest. 1970-80 was a golden time for the discovery and usage of restriction enzymes to cut DNA at specific sequences and introducing that sequence in host cells.

After these important discoveries, another valuable discovery was Double Strand Breaks (DBS) at a specific sequence, which revolutionized the genetic recombination technologies [2].

Genetic engineering approaches use the nucleases to cut the DNA. If we look at a brief history of mammalian genetic recombination than three classes of endonucleases, seem very important. All of these classes have the same action of working i.e. they produce a cut in both the strands of DNA at their respective sequence or site[3].

These classes include:

- Meganucleases (recognize long stretches).
- ZFN (zinc finger nucleases, recognize triple DNA code).
- TALEN (transcription activator-like effector nucleases, recognize original base).
- CRISPR (clustered regularly interspaced short palindromic repeats, determine PAM sequence)[4].
- These fragments produced are then ligated by two techniques.
- NHEJ (Non-homology end joining, result in small insertions or deletions in DNA).
- HDR (Homology directed repair, result in precise recombination)[2].

1.1 CRISPR-Cas system

It was discovered in 1987 and after the discovery of similar sequences in other microbes, the acronym was devised in 2002. These sequences are present as direct repeats that are separated by specific Spacers (variable sequence stretches between two repeat sequences). Spacer regions are responsible for coordinating with the foreign DNA inserted as either phage DNA, transposable elements or a plasmid [2].

CRISPR sequences are present along with CRISPR associated proteins called Cas genes which are present adjacent to the spacer regions. These associated sequences encode for the proteins like; nucleases, polymerases, helicases, and ligases.

CRISPR also has some other proteins associated called, repeat-associated mysterious proteins (RAMP) [5].

The size of CRISPR repeats is 23-21bp while the size of the spacer region is 47-72bp. Within a specific locus, the sequence remains conserved but varies in different species. Repeat-spacer units are up to 375 but the commonly present units are less than 50. This sequence may be present in more than one locus, which makes it a huge part of the total genome of microbes. The presence of CRISPR is mostly in the chromosomal DNA but it is also reported in plasmid DNA[6].

Crispr contains a leader sequence, which is present upstream to the first repeat and may act as a promoter sequence for the transcription of pre- CRISPR RNA. This RNA is then processed and cleaved to produce rather smaller cr RNA. The processing of the RNA may differ from specie to specie, i.e. in E.coli, proteins called Cascade CRISPR-associated complex for antiviral defense) cleaves the RNA while in Pyrococcus, proteins called Cas 6 act as the nuclease. All the CRISPR works along with Cas as inactivation of these proteins result in loss of immunity against phages [7].

1.2 History

CRISPR stands for “clustered regularly interspaced palindromic repeats”. The new endonuclease, which is the active research in genetic engineering, is CRISPR/ Cas [8]. It was discovered first in 1987 in E. coli during its genetic analysis, but due to limited resources at that time, the functions of these sequences were unknown. The first observation of the repeats was in Archaea in 1993, and preceding this it was also discovered in the bacterial genome in a conserved manner. The presence of a conserved sequence repeats in more than one domain was an astounding discovery [9]. The loci for CRISPR has been found in almost 90 of the archea and 40% of bacteria. During evolution, as a basis of adaptive immunity, this loci has been propagated into prokaryotes.

In 2005, it was reported that these sequences have spacer DNA similar to DNA of phages, which were thought to be involved in the adaptive immunity of these microbes. In 2007, further research was converged towards another endonuclease, which works along with CRISPR and explains the function much more simply, called the Cas 9 protein. After this, guide RNA was mixed with Cas 9 to make precise cuts in DNA, which made it a new genetic manipulation tool for the new era[2, 9].

1.3 *RNA-mediated immunity*

Microbes are always taking up foreign DNA either in their reproduction methods or by injection of phage DNA. To maintain the integrity of the genetic materials, it is necessary to be able to differentiate self-DNA from foreign DNA even when it has been integrated into the DNA. It can either be one by blocking the absorption of foreign DNA or by cleaving it through different methods. CRISPR is the newly discovered tool present in microbes. Microbe's immunity is related to the presence of CRISPR sequences, which can cleave and bind the DNA at specific sequences.

There are 3 basic steps of the adaptive immunity produced in microbes:

1. Adaptation: The foreign DNA injected by the phage is cleaved and integrated into the CRISPR array through the endonuclease that works along with the system.
2. Expression: The integrated DNA translate along with the original DNA making crRNA (CRISPR RNA) which interacts with Cas effector nuclease and assemble to form a surveillance complex.
3. Interference: The complex has endonuclease activity and the sequence of the phage DNA to guide the activity, so now when a phage attack, these complexes can provide adaptive immunity against it[9, 10].
4. It was shown in *Streptococcus thermophilus* in 2007 that by alteration of CRISPR sequences in the genome, phage resistance was produced. To justify this, the addition and deletion of specific spacer regions was done in the laboratory, which produced immunity and caused a loss of immunity respectively.

2. CLASSIFICATION

2.1. Class 1 CRISPR–Cas systems

The effector complex of this system is multi-subunit. It includes type 1, type 3 and type 4 Cas systems.

2.1.1. Type I CRISPR–Cas systems

They contain Cas 3 genes or their variants encoding a single-stranded DNA stimulated by helicase, which can show its activity on DNA-DNA and DNA-RNA interactions. It includes seven subtypes i.e. Type I A – F and Type I U.

2.1.2. Type III CRISPR–Cas systems

They contain Cas 10 genes, which encodes a multi-domain protein of which one domain is called a palm domain. All the loci also contain a rather smaller sub-unit having Cas 5 and Cas 7 genes. It includes 2 subtypes i.e. Type III-A and Type III B.

2.1.3. Putative type IV CRISPR–Cas systems

A reported and uncharacterized system is also present in the plasmids of the microbes called type IV. They are present far from the CRISPR array and lack Cas 1 and Cas 2 genes. They can make a surveillance complex but its larger subunit is mostly present in a partially degraded form.

2.2. Class 2 CRISPR–Cas systems

The surveillance complex formed by this system is a single subunit and includes 2 subclasses i.e. type II and type V.

2.2.1. Type II CRISPR–Cas systems

The gene present in this system is by far the simplest and most commonly used, called, Cas 9 protein, which can form a surveillance complex to cleave the target DNA. This system also contains Cas 1 and Cas 2 genes. The system is divided into 3 subtypes i.e. II-A and II-B and II-C.

2.2.2. Putative type V CRISPR–Cas systems

It contains a protein called cpf1, which combines with an adaptor molecule to make a single subunit surveillance complex [9].

2.2.3 CRISPR/Cas 9

Zinc finger nucleases were used as genetic editing tool until 2013 [11] along with TALENS [12]. The new era of genetic engineering started with the discovery of the CRISPR tool for genome editing. There are further classes of CRISPR out of which the most commonly used class is type 2 which work along the CRISPR associated protein called Cas 9 obtained from *Streptococcus pyogenes* [13].

The CRISPR system works by cleaving the foreign DNA by using the nuclease domains of the associated proteins in specific sequences only, these cleaved sequences are the integration between the repeat sequences as "spacers" [14]. The system works by the transcription of these regions in which the crRNA has both original and the foreign DNA transcribed into one. Further processing takes place by the interference of crRNA to TrcrRNA which will then make a complex with Cas 9 [15].

Cas 9 is a multi-subunit protein with two nuclease domains called HNH and RuvC like domains. The protein edit genome by producing double-stranded cuts by the interference of the two-nuclease domains [16]. Cas 9 will produce a cleavage in the foreign DNA at specific sequences called protospacers [14]. The recognition of the specific sequences is done by the presence of some conserved sequences that are present downstream to the DNA that is to be cleaved. These conserved sequences are called protospacer-adjacent motif (PAM) having a sequence of 5'-NGG-3' [13, 17] but in rare cases may also be NAG [18]. The cleavage produced by these nucleases are highly specific and this is due to the presence of Seed Sequences that are present upstream to the PAM sequence. This sequence must be complementary between the DNA and RNA for the specificity [19].

3. CRISPR/CAS9 SPECIFICITY

Initially, a 20nt sequence presence in gRNA was considered important for a sequence-specific cut but later on, it was discovered that the presence of a smaller sequence upstream to the DNA that is to be targeted is important for specific cleavage, and was called the Seed Sequence [13, 20, 21]. There is needed a perfect base pairing between the RNA (gRNA) and DNA to produce a specific cut but in case of presence of an extra-base in DNA will cause the production of a bulge in DNA while in case of a missing base the bulge will be produced in the gRNA which can result in an off-target cleavage [22].

To increase specificity, there are many strategies applied to the system. The easiest and used strategy is to design gRNA accordingly to guide a specific cut in the DNA. There are bioinformatic tools available for designing (i.e CHOPCHOP (https://chopchop.rc.fas.harvard.edu) http://tools.genome-engineering.org, http://zifit.partners.org, and www.e-crisp.org) [23, 24, 25]. As CRISPR work on complete base pairing for specificity, sequence analysis can be done to give higher predictability [26]. Also, this system is very easily re-programmed, so by trial and error, accurate results can be obtained rather easily [18]. Another way to lower the off-target cleavage is to control the expression of the nuclease as a higher concentration of Cas 9 and gRNA can also be a reason for lower specificity [18, 27, 28].

In the case of mutation in the ruvC domain can mutate it to produce a single cut (nick) production. A pair of sgRNA is used to guide Cas 9 nuclease to produce a single-stranded cut in both the strands of DNA simultaneously will produce a double-stranded cut with staggered ends [8]. By this strategy, precise cleavage can be produced in both the DNA strands as compared to the wild type of Cas 9 i.e. non-mutated. This strategy has been proved to be more precise in human and mice models [29, 30, 31]. Although this has proved a very precise strategy, it can still cause other mutations. To overcome this problem, mutated Cas 9 was fused with FokI nucleases, which lower the mutation rate and increase the specificity of cuts simultaneously [32, 33].

Another strategy is to add extra G residues at the 5' end that can lower the chances of off-target cleavage but at the same time make them less active for actual cleavage while truncated gRNA lowered mutation and were more active at the target site. Truncated gRNA can also be complexed along with Cas 9 for more sensitivity [34].

The rate of mutation can differ from cell to cell within a specie. For instance, based on WES the rate of mutations in cancer line cells is higher in comparison to pluripotent stem cells of humans [35, 36, 37]. Similarly in rice, 11bp upstream to PAM a single nucleotide was mismatched and the rate was 5 times lower than that of a target mutation but when a seed sequence was provided then no off-target mutation was observed [38-43].

4. APPLICATIONS

4.1 Genome editing

The most feasible tool for editing the genome, being used from 2013 is CRISPR. The most common way can be by adding a vector (like plasmid) having CRISPR/Cas 9 along with the crRNA expression which can produce the desired mutation in the genome [44, 45].

The gene-editing has been done in multiple mammalian species and others i.e. humans, [46] drosophila, [47, 48, 49] zebrafish [50] Bombyx mori [51] Caenorhabditis Elegans [52] and bacteria [53, 54]. Many mutations can be produced simultaneously in different species only by the addition of different gRNA which makes this editing more feasible. Gene editing can also be done in many plants to add or delete specific characters i.e. in tobacco, Arabidopsis [55] and rice [56].

4.2 Delivery methods

Different carriers are used to deliver the desired material into the host cells. The induction of Cas9 in the target cells is a very important step for gene editing. The pay-load should not be degraded by the body and it must enter the nucleus of the cell. Cargo can be in the form of DNA, protein/ ribonucleoprotein or RNA. All these steps are dependent on the delivery method and the carrier used [57]. Following are some important carriers for Cas9 that are being used in the laboratories:

4.3 Viruses

Viruses have been proved to be a promising delivery system as they can invade cells efficiently and can induce the production of the desired gene inside the host. If the replication gene is removed and replaced by any other transgene so that expression is produced without any replication in other cells [58]. AAV (adeno-associated vectors) are used most frequently for targeted delivery [59-61] but the maximum limit of DNA encapsulation of the virus is as low as 4.7kb thus the gRNA and Cas9 should be added in two different AAVs [62].

4.4 Non-viral

Many non-viral delivery methods are being used which include physical methods, chemical modifications and encapsulation methods [63]. These methods give more control to the delivery in sense of dosage, specificity, etc.

4.5 Physical

This technique induces a disruption in the physical barriers and a direct delivery to the target destination. It can be done by electroporation (in vitro) which uses an electric field to make the cell membrane porous temporarily so that DNA, RNA or protein material (bulky biomolecules) can enter the cell directly [64, 65].

Another technique is hydrodynamic injections, which are an in vivo technique in which an intravenous injection injects a liquid at high pressure and volume, which makes temporary pores and biomolecules can enter the target. Until now, this technique is used only for small animals like mice in which an injection in the tail was used to inject the plasmids in heart, kidney, lungs and liver tissues [66, 67, 68].

Similarly, microinjections can also be used to make a small piercing in the cell membrane and injecting the cargo directly in the cell [69].

4.6 Chemical method

It can be done either directly by the chemical modification i.e Cell-penetrating peptide combined with a nuclear localization signal in the cargo or by encapsulation of the cargo so that it's not degraded by the body.

4.7 Encapsulation

Encapsulation methods can be used in the form of liposomes [70]. An important lipid delivery system is lipofectamine [71, 72]. This method can also use the encapsulation of polymers (biological/synthetic) [73]. Recently, nucleic acid polymers are also been explored to be used as capsules for the delivery of Cas9 [63]. Many

nanoparticles are also used for delivery purposes i.e. gold nanoparticles, carbon nanoparticles, and silica nanoparticles. Among these, gold nanoparticles are used more frequently for the in vivo transfer of Cas9[74].

5. REGULATION OF TRANSCRIPTION

Expression of a gene can be regulated at the transcription level but the response produced by this will be irreversible. CRISPR/Cas 9 work by changing/disrupting the sited that control the transcription of a specific gene. A modified system is designed by this and is called CRISPR Interference i.e CRISPRi [76, 77].

When mutated Cas 9 were complexed with activating and repressing domains along with gRNA, a precise activation and repression of a gene were observed due to regulation of transcriptional genes [78]. Similarly, in another research using CRISPri, multiple gene activity was altered by regulating their transcriptional activity simultaneously. This system provides a novel approach to regulate gene expression without any alteration in the DNA [76].

6. GENE THERAPY

Gene therapy is the most advanced level of treatment that can cure the endogenous disease permanently by genome editing. This can be done by either removing the disease-causing gene or by adding some other gene to overcome or protect the effect of disease-causing mutation [79-81].

In a mouse model by the gene-editing by CRISPR/Cas9 in DMD gene of the germline, almost all the somatic cells lost the disease-causing genes [82].

In HIV infected cells, promotor of HIV-1 was cleaved by Cas9, which caused a lower expression of HIV. By a similar method, those viral genes that are already integrated into the genome can be cleaved to remove the infection causing genes by the help of gRNA [83].

Induced pluripotent stem (iPS) cell production is also using the CRISPR system for editing of genome. When Cas9 is inserted along with gRNA, in a study, the DNMT3B gene was disrupted. As this gene on mutation was producing immunodeficiency, facial anomalies syndrome and centromeric instability, a disruption in the mutated gene caused a significantly lower expression of mutation [84]. Similar results have also been obtained from patients affected by β-thalassemia [85].

Many viral infections are also related to the production of cancer. In these cases, by removing these genes, cancer can be treated with gene therapy. For instance, cervical carcinoma lines of cells that are HPV-positive and Burkitt's lymphoma line of cells that are EBV-positive have lost their proliferation and viral activity by CRISPR gene therapy [86].

In other types of cancer (non-viral) that are caused by genetic mutations. CRISPR can edit the genome at genetic [87] and epigenetic level [88] to edit out the mutations. The mutated gene can also be switched off by regulating the transcription in the modified model of CRISPRi.

7. APPLICATIONS OTHER THAN GENOME EDITING

CRISPR system can also work by regulating the gene expression without directly editing the genome. It can bind to the proteins that are involved in gene expressions like polymerases or transcription factors. CRISPRi modification is based upon this binding of dCas, which can cause a knockout expression of a gene by controlling the transcription [77].

When a mutation takes place in Cas9 and instead of double-stranded break, only a nick is produced in the DNA called nickase Cas9. This tool is used to directly replace A/T and G/C [89]. In a recent study, it was reported that when nickase Cas 9 is complexed with APOBEC1 deaminase and UGI, there is no DSB produced and C is converted to T [90]. A can also be converted to G by a novel complex of nickase with adenosine deaminase [91]. By changing one base to another, amino acid coding codon can also change into stop codon by which the protein formation can be altered by the presence of an early stop codon [92].

CRISPR also plays an important role in epigenetic regulation. The most common ways of regulation are DNA methylation, acetylation and modifications of histone and among these, the most common way is DNA methylation [93]. dCas9 is used for locus-specific methylation by fusion of dCas9 with DNA methyltransferase enzyme [94]. Also, by a similar approach, DNA demethylation is done by an enzyme called ten-eleven

translocation (TET) proteins. By fusion of these proteins with dCas9 produced more specific results and active transcription of the genes [95].

Another important application of CRISPR is chromatin imaging of the live cell. dCas loaded with fluorescent proteins were targeted to the repeat regions of the DNA to produce chromatin image [96]. With a similar approach, repeat regions of telomeres, as well as centromere, were targeted by dCas9 (labeled) to create a contrast image of chromatin [97].

From an abscisic acid pathway, (plant) two protein domains that can make dimers i.e. ABI1 and PYL1 were taken and fused with dCas9, which forced the dimerization of the two proteins, which lead to a formation of a loops-like structure between promotor and enhancer region. This loop (induced) increased the expression of β-globin in hematopoietic cells[97].

8. CONCLUSIONS

CRISPR is a relatively new technique for genetic engineering. Although there are many techniques that are being used already that are described here but still there are advances that are being developed. The basic concern is the off-target cuts by the system. Each technique described here also have shortcomings of their own, which should be overcome by new adaptations. This system is still evolving, and many modifications of the system are developing making the system to adapt to the new techniques. CRISPR is very sensitive technique so to obtain specific results special care must be taken to handle the system.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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